DOE CASE S-103,779

MODIFIED CELLULOSE SYNTHASE GENE FROM *ARABIDOPSIS THALIANA* CONFERS HERBICIDE RESISTANCE TO PLANTS

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PROVISIONAL PRIORITY AND U.S. GOVERNMENT RIGHTS

This application is a continuation-in-part of, and seeks priority to, application No. 09/686,234 filed on Oct. 11, 2000, which in turn was submitted in reference to, and sought priority of, Provisional Application No. 60/159,369 filed on October14, 1999 that bears an identical title. The United States Government has rights in this invention pursuant to Contract No. DE-FG02-94ER20133 between the U.S. Department of Energy and the Carnegie Institute of Washington.

Sequence Listing

The contents of the paper and computer readable copies of the sequence listing submitted herewith are the same.

Field of the invention

This invention relates to a mutant gene coding for isoxaben and thiazolidinone-resistant cellulose synthase ("CS"), and the process of imparting such resistance to a plant crop.

Background of Invention

The ability to modify microorganisms and cells of higher organisms by genetic engineering has made it possible to change certain of their specific characteristics and thereby alter the response of those organisms to various agents. Of particular interest are the responses of organisms to agents used because of their cytotoxic effect. For example, many compounds used in agriculture are directed to the killing of pests, weeds, or the like. Often these compounds can have a relatively long residence time or extended residue in the plants subjected to treatment by the compound.

In many situations it is desirable to differentiate the species to be retained from the species to be killed. For example, it is often necessary to selectively destroy weeds, yet have minimal impact on the economically valuable crop plants. For the most part, broad-spectrum herbicides have a sufficiently adverse effect on crops that their use must be limited to emergent use or careful postemergent application.

Some weed species are simply resistant to today's herbicides, increasing the importance of developing the production of effective herbicides. Moreover, as some weed species are controlled, competition is reduced for the remaining tenacious weed species. The development of genetically engineered herbicide-resistant crop plants could significantly improve weed-control by allowing fields to be treated with a single, concentrated application of the herbicide. Therefore, a one-step procedure could eliminate costly and perhaps ineffective repeated low-dosage herbicidal treatments, such as have been required in the past to avoid damaging conventional crops, but which may have also induced the emergence of spontaneous herbicide-resistant weeds. Herbicides with greater potency, broader weed spectrum and more rapid degradation after application would avoid the problematic persistence of the chemical herbicide in the soil, such as typically results from frequently repeated applications, and which prevents rotation of crops sensitive to that herbicide.

Certain herbicides, while not used directly to control weeds in field crops, are used as `total vegetation control agents` to eliminate weeds entirely in certain right-of-way or industrial situations. However, these herbicides may be deposited by natural means, such as water run-off, onto areas where economically important crops are growing. As a result sensitive field crops may be killed or their growth seriously inhibited. It is therefore highly desirable to be able to modify viable cells to make them resistant to stressful cytotoxic agents.

Isoxaben (–3[1-ethyl-1-methylpropyl]-5-isoxazolyl-2,6,dimethoxybenzamide), 2,6-dichlorobenzonitrile (DCB) and thiazolidinones such as 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidnone (TZ), are structurally diverse herbicides. On the basis of biochemical studies of mode of action, their primary target site has been proposed to be the enzyme cellulose synthase, which catalyses the synthesis of cellulose, a major component of plant cell walls. However, the precise target for these herbicides has not been previously described.

Isoxaben (EL-107, Flexidor, Gallery) is a preemergence, broad leaf herbicide used primarily on small grains, turf and ornamentals. The compound is extremely active with an I_{50} for *Brassica napus* of 20 nM (Lefebvre et al., 1987). Isoxaben inhibits the incorporation of glucose into the cellulose-rich, acid-insoluble fraction of isolated walls and is an extremely powerful and specific inhibitor of cell wall biosynthesis (Heim et al., 1990b; Corio-Costet et al.,1991b). Cell wall-fractionation studies have revealed that the herbicidal action of isoxaben can be explained entirely by its effect on cellulose biosynthesis (Heim et al., 1991). Its probable mode of action is to directly inhibit cellulose synthesis, because resistant cell lines show an unaltered uptake or detoxification of the herbicide (Heim et al., 1991) and only two genetic loci in *Arabidopsis thaliana*, termed *ixr*A (=*ixr*1) and *ixr*B (=*ixr*2), have been shown to confer resistance (Heim et al., 1989, 1990a). Exhaustive studies have revealed that other cellular processes are unaffected by isoxaben (e.g. seed germination, mitosis, respiration, photosynthesis, and lipid and RNA synthesis, Lefebvre et al., 1987; Corio-Costet et al., 1991a). Treated cells fail to elongate with high fidelity and consequently grow isodiametrically (Lefebvre et al., 1987). This herbicide acts at much lower concentrations (< 40 x) than dichlobenil, another cellulose synthesis inhibitor (Heim et al., 1990b). Therefore, the properties of isoxaben make it an ideal agent for perturbing the mechanical properties of the primary cell wall.

Thiazolidinones such as 5-tert-butyl-carbamoyloxy-3-(3-trifluromethyl) phenyl-4-thiazolidnone (TZ) are a new class of N-phenyl-lactam-carbamate herbicides (Sharples et al, 1998). TZ shows potential for selective preemergence control of a range of weed species in soybean and other crops. Susceptible weeds include grasses such as *Digitaria* spp., *Setaria* spp., *Sorghum* spp., and small seeded broad leafed weeds which include *Amaranthus* spp., and *Chenopodium* spp. TZ has a similar syndrome of effects on plants as isoxaben. A common mode of action with isoxaben is indicated by the fact that the ixrA1 (=*ixr*1-1) mutant of *Arabidopsis* exhibits resistance to both isoxaben and TZ (Sharples et al., 1998).

The herbicide 2,6-dichlorobenzonitrile (dichlobenil, DCB) is an effective and specific inhibitor of cellulose synthesis in algae and plants (Delmer et al., 1987). It has been reported to bind to an 18 kd polypeptide in cotton fiber extracts but no mechanism for its action has been demonstrated and no function for the 18 kd protein has been reported.

A crop made more resistant to isoxaben and thiazolidinone herbicides offers a selective means to control and kill weeds without adversely affecting the crop plant. Clearly then, an understanding of the method by which weeds become resistant to herbicides at the molecular level is essential to establishing a basis for the development of sound weed control programs. The molecular basis underlying the expression of isoxaben and thiazolidinone-resistance had remained undetermined until the present invention. Therefore, identification of the mutation site(s) in the CS gene, which code for the mutant plant's isoxaben and thiazolidinone resistance is of agricultural significance. The isolation of a mutant CS gene, which confers resistance to isoxaben and thiazolidinone in higher plants, would provide an opportunity to introduce isoxaben and thiazolidinone resistance into crop plants by genetic engineering. Isoxaben and thiazolidinones, because of their broad-spectrum activity and low mammalian toxicity, are particularly suited as a type of herbicide to which genetically engineered resistance would be economically important in crop plants. The development of isoxaben and thiazolidinone-resistant crops would provide a reliable and cost-effective alternative to conventional weed management programs.

By modifying crop plant cells by the introduction of a functional gene expressing the isoxaben and thiazolidinone-resistant CS enzyme, one can use isoxaben and thiazolidinones or an analogous herbicide with a wide variety of crops at a concentration which ensures the substantially complete or complete removal of weeds, while leaving the crop relatively unaffected. In this manner, substantial economies can be achieved in that fertilizers and water may be more efficiently utilized, and the detrimental effects resulting from the presence of weeds avoided.

The genetic code is degenerate, meaning that more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any set of similar DNA oligonucleotides. With respect to nucleotides, therefore the term "derivative(s)" is intended to encompass those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid.

SUMMARY OF THE INVENTION

It is a primary object of this invention to provide isoxaben and thiazolidinone-resistance, nucleic acid sequences encoding isoxaben and

thiazolidinone-resistant CS enzymes, constructs containing the genes coding for such isoxaben and thiazolidinone-resistant CS enzymes under the transcriptional and translational control of regulatory genes recognized by a desired host to which the isoxaben and thiazolidinone-resistant CS enzyme genes are foreign, host cells containing such constructs, and organisms and organism parts or products containing such constructs.

It is an additional object of this invention to provide a gene coding for isoxaben and thiazolidinone-resistant CS enzymes useful in the transformation of a crop plant, and thereby effective in the protection of the host cells from the cytotoxic effect of isoxaben and thiazolidinone.

It is also an object of this invention to provide a novel mutant CS gene useful in the transformation of crop plants, wherein the isoxaben and thiazolidinone resistance of the transformed crop plant is greater than that of an untreated isoxaben and thiazolidinone-sensitive wild-type crop plant.

It is a particular object of this invention to provide a novel CS gene capable of coding for sufficient isoxaben and thiazolidinone resistance in a transformed crop plant that, after planting, sufficiently concentrated isoxaben and thiazolidinone herbicide can be applied in a single treatment to the transgenic plant crop to selectively kill substantially all of the weeds, without application of an additional herbicide.

It is further an object of this invention to provide a method of producing a transformed plant crop that, after planting, exhibits greater resistance to isoxaben and thiazolidinone herbicide than that of an isoxaben and thiazolidinone-sensitive wild-type crop plant of the same species.

It is a further object of this invention to increase the effectiveness of isoxaben and thiazolidinone herbicides by producing plant crops less susceptible to damage by these herbicides when used to control weeds.

It is a further object of this invention to provide isoxaben and thiazolidinone-resistant CS enzyme gene constructs useful in selectively distinguishing between host cells containing the construct and host cells lacking such construct.

It is a further object of this invention to provide a method for the rational design of novel chemical compounds that inhibit the activity of plant cellulose synthases and are, therefore, useful as novel herbicides. The knowledge of the site of inhibitory action of the

isoxaben and thiazolidinone herbicides disclosed here, in conjunction with knowledge of which amino acid residues can be changed to impart resistance to the inhibitory action of the herbicides enables the design of additional herbicidal compounds by methods familiar to those skilled in the art of rational chemical design.

Another aspect of this invention is an isolated and purified nucleic acid comprising the nucleic acid sequence of (SEQ ID NO: 1).

Another aspect of this invention is an isolated and purified nucleic acid comprising the nucleic acid sequence of (SEQ ID NO: 2).

Another aspect of this invention is the isolated and purified nucleic acids comprising other nucleic acid sequence cited or envisioned by this disclosure.

These and other objects are accomplished pursuant to the practice of this invention.

DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the relative position of the molecular markers on chromosome V that were used to map the *ixr*1 gene. Names of BAC-clones are indicated above the line and names of molecular markers are given below the lines. Numbers in brackets are the size of the non-overlapping sequence for each BAC-clone, or the number of recombinants found for each marker

DESCRIPTION OF PREFERRED EMBODIMENTS

Definitions

Before proceeding further with a description of the specific embodiments of the present invention, a number of terms will be defined.

As used herein, a "compound" or "molecule" is an organic or inorganic assembly of atoms of any size, and can include macromolecules, peptides, polypeptides, whole proteins, and polynucleotides.

The determination of percent identity or homology between two sequences is accomplished using the algorithm of Karlin and Altschul (1990) Proc. Nat'l Acad. Sci. USA 87: 2264-2268, modified as in Karlin and Altschul (1993) Proc. Nat'l Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs

of Altschul et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches are performed with the NBLAST program, score=100, wordlength=12 to obtain nucleotide sequences homologous to the nucleic acid molecules of the invention. BLAST protein searches are performed with the XBLAST program, score=50, wordlength=3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST is utilized as described in Altschul et al. (1997) Nucleic Acids Res. 25: 3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) are used. See http://www.ncbi.nlm.nih.gov.

As used herein, a "polynucleotide" is a nucleic acid of more than one nucleotide. A polynucleotide can be made up of multiple poly-nucleotide units that are referred to be description of the unit. For example, a polynucleotide can comprise within its bounds a polynucleotide(s) having a coding sequence(s), a polynucleotide(s) that is a regulatory region(s) and/or other polynucleotide units commonly used in the art.

The isolated nucleic acid molecule of the present invention can include a deoxyribonucleic acid molecule (DNA), such as genomic DNA and complementary cDNA which can be single (coding or noncoding strand) or double stranded, as well as synthetic DNA, such as synthesized single stranded polynucleotide. The isolated nucleic acid molecule of the present invention can also include a ribonucleic acid molecule (RNA).

As used herein, the terms "hybridization" (hybridizing) and "specificity" (specific for) in the context of nucleotide sequences are used interchangeably. The ability of two nucleotide sequences to hybridize to each other is based upon a degree of complementarity of the two nucleotide sequences, which in turn is based on the fraction of matched complementary nucleotide pairs. The more nucleotides is a given sequence that are complementary to another sequence, the greater the degree of hybridization of one to the other. The degree of hybridization also depends on the conditions of stringency which include: temperature, solvent ratios, salt concentrations, and the like.

In particular, "selective hybridization" pertains to conditions in which the degree of hybridization of a polynucleotide of the invention to its target would require complete or nearly complete complementarity. The complementarity must be sufficiently high as to assure that the polynucleotide of the invention will bind specifically to the target relative to binding other nucleic acids present in the hybridization medium. With selective

hybridization, complementarity will be 90-100%, preferably 95-100%, more preferably 100%.

The term "stringent conditions" is known in the art from standard protocols (e.g. Current Protocols in Molecular Biology, editors F. Ausubel et al., John Wiley and Sons, Inc. 1994) and is when hydridization to a filter-bound DNA in 0.5M NaHPO.sub4 (pH7.2), 7% sodium dodecyl sulfate (SDS), 1mM EDTA at +65°C, and washing in 0.1XSSC/0.1%SDS at +68°C.

Degenerate variant is the redunency or degeneracy of the genetic code as is well known in the art. Thus the nucleic acid sequences shown in the sequence listing provided only examples within a larger group of nucleic acids sequences that encode for the polypeptide desired.

"Isolated" nucleic acid will be nucleic acid that is identified and separated from contaminant nucleic acid encoding other polypeptides from the source of nucleic acid. The nucleic acid may be labeled for diagnostic and probe purposes, using any label known and described in the art as useful in connection with diagnostic assays.

Because the genetic code is degenerate, more than one codon may be used to encode a particular amino acid, and therefore, the amino acid sequence can be encoded by any set of similar DNA oligonucleotides. With respect to nucleotides, therefore, the term "derivative(s)" is also intended to encompass those DNA sequences which contain alternative codons which code for the eventual translation of the identical amino acid.

Nucleotide Sequences

The scope of the present invention is not limited to the exact sequence of the cDNA sequences set forth in (SEQ ID NO: 1), (SEQ ID NO:2) and (SEQ ID NO: 3) or the use thereof. The invention contemplates certain modifications to the sequence, including deletions, insertions, and substitutions, that are well known to those skilled in the art. For example, the invention contemplates modifications to the sequence found in (SEQ ID NO: 4) and (SEQ ID NO: 6) that encode the same amino acid substitutions as found in (SEQ ID NO: 1) and (SEQ ID NO: 3). Similarity it is expected that other sequence having codons that encode amino acids that are chemically equivalent to the amino acids substituted by (SEQ ID NO: 1) and (SEQ ID NO:2) would have the same resistance effect.

Creating various the point mutation variation envisioned by this invention can be

accomplished by a variety of protocols known in the art including those described in U.S. Patent No. 6,448,048 issued to Tomono et al., on Sep. 20, 2002.

Chemical equivalency can be determined by one or more the following characteristics: charge, size, hydrophobicity/hydrophilicity, cyclic/non-cyclic, aromatic/non-aromatic etc. For example, a codon encoding a neutral non-polar amino acid can be substituted with another codon that encodes a neutral non-polar amino acid, with a reasonable expectation of producing a biologically equivalent protein.

Amino acids can generally be classified into four groups. Acidic residues are hydrophillic and have a negative charge to loss of H+ at physiological pH. Basic residues are also hydrophillic but have a positive charge to association with H+ at physiological pH. Neutral nonpolar residues are hydrophobic and are not charged at physiological pH. Neutral polar residues are hydrophillic and are not charged at physiological pH. Amino acid residues can be further classified as cyclic or noncyclic and aromatic or nonaromatic, self-explanatory classifications with respect to side chain substituent groups of the residues, and as small or large. The residue is considered small if it contains a total of 4 carbon atoms or less, inclusive of the carboxyl carbon. Small residues are always non-aromatic.

Of naturally occurring amino acids, aspartic acid and glutamic acid are acidic; arginine and lysine are basic and noncylclic; histidine is basic and cyclic; glycine, serine and cysteine are neutral, polar and small; alanine is neutral, nonpolar and small; threonine, asparagine and glutamine are neutral, polar, large and nonaromatic; tyrosine is neutral, polar, large and aromatic; valine, isoleucine, leucine and methionine are neutral, nonpolar, large and nonaromatic; and phenylalanine and tryptophan are neutral, nonpolar, large and aromatic. Proline, although technically neutral, nonpolar, large, cyclic and nonaromatic is a special case due to its known effects on secondary conformation of peptide chains, and is not, therefore included in this defined group.

There are also common amino acids which are not encoded by the genetic code include by example and not limitation: sarcosine, beta-alanine, 2,3 diamino propionic and alpha-aminisobutryric acid which are neutral, nonpolar and small; t-butylalanine, t-butylglycine, –methylisoleucine, norleucine and cyclohexylalanine which are neutral, nonpolar, large and nonaromatic; ornithine which is basic and noncylclic; cysteic acid

which is acidic; citrulline aceyl lysine and methionine sufoxide which are neutral, polar, large and nonaromatic; and phenylglycine, 2-napthylalanine, ß-2thienylalanine and 1,2,3,4, tetrahydroisoquinoline-3cboxylic acid which are neutral, nonpolar, large and aromatic. Other modifications are known in the art some of which are discussed in U.S. Patent 6,465,237 issued to Tomlinson on Oct. 15, 2002.

This invention embodies the isolation and purification of novel mutant CS genes from isoxaben and thiazolidinone-resistant plants, sequencing to identify the unique mutations, and transforming an isoxaben and thiazolidinone-sensitive plant to confer greater isoxaben and thiazolidinone resistance than that originally possessed by the transformed plant.

The novel CS gene of interest may be obtained from a higher plant, particularly a plant shown capable of resisting isoxaben and thiazolidinone treatment. The mutant plant can be the result of a spontaneous mutation or to various mutagenic processes, including chemical, biological, radioactive, or ultraviolet treatments.

The gene encoding the isoxaben and thiazolidinone-resistance CS enzyme may be cloned by map based cloning, as described herein. As a preferable alternative, the gene encoding the isoxaben and thiazolidinone-resistance CS enzyme may be cloned by using the corresponding gene from *Arabidopsis* or another plant as a heterologous hybridization probe to isolate genomic or cDNA clones of the gene from another plant, or oligonucleotides based on the CS gene nucleotide or polypeptide sequence may be used to isolate all or part of the gene by PCR and these fragments may then be cloned by standard procedures familiar to those skilled in the art.

Depending on the size of the identified fragment, it will usually be further manipulated so that it contains the full coding sequence of the CS gene and its flanking regulatory sequences. Partial cleavage with different restriction enzymes in different reaction mixtures may be employed, followed by cloning of the fragments to determine which of the fragments still retain the ability to provide isoxaben and thiazolidinone-resistant CS activity.

The gene coding for isoxaben and thiazolidinone-resistant CS activity may be modified in a variety of ways, truncating either or both of the 5'- or 3'-termini, extending the 5'- or 3'-termini, or the like. Usually, not more than 25, in particular not more than about 20

codons will be involved in the modification of the naturally occurring isoxaben and thiazolidinone-resistant CS gene. The gene may be extended by as many as 50 amino acids, usually not more than about 30 amino acids. Combinations of substitution, truncation and extension may be employed. Thus the gene may be manipulated in a variety of ways to change the characteristic of the enzyme, for convenience in manipulation of the plasmids, or the like. Based on knowledge of the molecular basis of isoxaben and thiazolidinone-resistance, similar mutations could be introduced by various site directed mutagenesis procedures, or by the production of partially or completely synthetic genes, into other plant CS genes to obtain isoxaben and thiazolidinone-resistance.

The DNA sequence containing the structural gene expressing the isoxaben and thiazolidinone-resistant CS may be joined to a wide variety of other DNA sequences for introduction into an appropriate host cell. The companion sequence will depend upon the nature of the host and the manner of introduction of the DNA sequence into the host.

For prokaryotic hosts, a wide variety of vectors exist which may be used for introduction by transformation, conjugation, transduction or transfection of the DNA sequence into a prokaryotic host. DNA vectors include a wide variety of plasmids, such as pBR322, pMB9, and the like; cosmids, such as pVK100; or viruses, such as P22, and the like.

For eukaryotic hosts, a wide variety of techniques may be employed for DNA introduction into the host, such as transformation with Ca^{++} -precipitated DNA, involving a non-replicating DNA sequence, a plasmid or a minichromosome, transformation, microinjection with a micropipette, electroporation, polyethylene glycol (PEG) mediated transformation of protoplasts, or gene gun or particle bombardment techniques. Whether the DNA may be replicated as an episomal element, or whether the DNA may be integrated into the host genome and the structural gene expressed in the host, will be determined by the presence of a competent replication system in the DNA construction. Episomal elements may be employed, such as tumor inducing plasmids, e.g., Ti or Ri, or fragments thereof, or viruses, e.g., CaMV, TMV or fragments thereof, which are not lethal to the host, and where the structural gene is present in such episomal elements in a manner allowing for expression of the structural gene. Of particular interest are fragments

having the replication function and lacking other functions such as oncogenesis, virulence, and the like.

To introduce isolated genes or groups of genes into the genome of plant cells an efficient host gene vector system is necessary. The foreign genes should be expressed in the transformed plant cells and stably transmitted, somatically or sexually to a second generation of cells produced. The vector should be capable of introducing, maintaining, and expressing a gene from a variety of sources in the plant cells. Additionally, it should be possible to introduce the vector into a variety of plants, and at a site permitting effective gene expression. Moreover, to be effective, the selected gene must be passed on to progeny by normal reproduction.

The fragments obtained from the isoxaben and thiazolidinone-resistant source may be cloned employing an appropriate cloning vector. Cloning can be carried out in an appropriate unicellular microorganism, e.g., a bacterium, such as *E. coli*, or *Salmonella*. In particular, one may use a phage, where partial or complete digestion provides fragments having about the desired size. For example, the phage lambda may be partially digested with an appropriate restriction enzyme and ligated to fragments resulting from either partial or complete digestion of a plasmid, chromosome, or fragment thereof. Packaging will insure that only fragments of the desired size will be packaged and transduced into the host organism.

The isoxaben and thiazolidinone-resistant CS enzyme may be expressed by any convenient source, either prokaryotic or eukaryotic, including bacteria, yeast, filamentous fungus, animal cells, plant cells, etc. Where secretion is not obtained, the enzyme may be isolated by lysing the cells and isolating the mutant CS according to known ways. Useful ways include chromatography, electrophoresis, affinity chromatography, and the like.

The DNA sequence encoding for the isoxaben and thiazolidinone-resistant CS activity may be used in a variety of ways. The DNA sequence may be used as a probe for the isolation of mutated or wild type CS sequences. Also saturation or site-directed mutagenesis could be performed on a plant CS gene to select for mutants expressing greater levels of herbicide-resistance, as well as resistance to additional classes of herbicide. Alternatively, the DNA sequence may be used for integration by recombination into a host to provide isoxaben and thiazolidinone resistance in the host. The mutant CS

gene can also be used as selection marker in the plant transformation experiments using the isoxaben and thiazolidinone herbicide as the selection agent.

With plant cells, the structural gene as part of a construction may be introduced into a plant cell nucleus by micropipette injection for integration by recombination into the host genome. Alternatively, methods including electroporation, polyethylene glycol (PEG) mediated transformation of protoplasts, or gene gun or particle bombardment techniques may be employed for introduction of the structural gene into a plant host cell. Where the structural gene has been obtained from a source having regulatory signals, which are not recognized by the plant host, it may be necessary to introduce the appropriate regulatory signals for expression. Where a virus or plasmid, e.g., tumor inducing plasmid, is employed and has been mapped, a restriction site can be chosen which is downstream from a promoter into which the structural gene may be inserted at the appropriate distance from the promoter. Where the DNA sequences do not provide an appropriate restriction site, one can digest for various times with an exonuclease, such as Bal31 and insert a synthetic restriction endonuclease site (linker).

Directed genetic modification and expression of foreign genes in dicotyledons (broad-leaf plants) such as tobacco, *Arabidopsis* etc. has been shown to be possible using the T-DNA of the Ti plasmid of *Agrobacterium tumefaciens*. Following genetically engineered insertion of a foreign DNA fragment into T-DNA in Agrobacterium, the host plant can be transfected by the bacterium or Ti plasmid, thus inserting the foreign DNA into the host plant chromosome to eventually produce a genetically engineered plant. Alternatively Ri, or root-inducing, plasmids may be used as the gene vectors. Although *Agrobacterium* effectively transform only dicots, the Ti plasmid permits the efficacious manipulation of the bacteria to act as vectors in monocotyledonous crop plants, i.e., wheat, barley, rice, rye, etc. Alternatively, Ti plasmids or other plasmids may be introduced into the monocots by artificial methods such as microinjection, or fusion between the monocot protoplasts and bacterial spheroplasts containing the T-region which could then be integrated into the plant nuclear DNA.

By employing the T-DNA right border, or both borders, where the borders flank an expression cassette comprising the isoxaben and thiazolidinone-resistant CS structural gene under transcriptional and translational regulatory signals for initiation and termination

recognized by the plant host, the expression cassette may be integrated into the plant genome and provide for expression of the isoxaben and thiazolidinone-resistant CS enzyme in the plant cell at various stages of differentiation. Various constructs can be prepared providing for expression in plant cells.

To provide for transcription, a variety of transcriptional initiation regions (promoter regions), either constitutive or inducible, may be employed. The transcriptional initiation region is joined to the structural gene encoding the isoxaben and thiazolidinone-resistant CS activity to provide for transcriptional initiation upstream from the initiation codon, normally within about 200 bases of the initiation codon, where the untranslated 5'-region lacks an ATG. The 3'-end of the structural gene will have one or more stop codons which will be joined to a transcriptional termination region functional in a plant host, which termination region may be associated with the same or different structural gene as the initiation region.

The expression cassette is characterized by having the initiation region, the structural gene under the transcriptional control of the initiation region, and the termination region providing for termination of transcription and processing of the messenger RNA, in the direction of transcription as appropriate.

Transcriptional and translational regulatory regions, conveniently tml promoter and terminator regions from A. tumefaciens may be employed, which allow for constitutive expression of the isoxaben and thiazolidinone-resistant CS gene. Alternatively, other promoters and/or terminators may be employed, particularly promoters which provide for inducible expression or regulated expression in a plant host. Promoter regions which may be used from the Ti-plasmid include opine promoters, such as the octopine synthase promoter, nopaline synthase promoter, agropine synthase promoter, mannopine synthase promoter, or the like. Other promoters include viral promoters, such as CaMV Region VI promoter or full length (35S) promoter or the like.

The various sequences may be joined together in conventional ways. The promoter region may be identified by the region being 5' from the structural gene, for example, the tml gene, and may be selected and isolated by restriction mapping and sequencing. Similarly, the terminator region may be isolated as the region 3' from the structural gene. The sequences may be cloned and joined in the proper orientation to

provide for constitutive expression of the isoxaben and thiazolidinone-resistant CS gene in a plant host.

The expression cassette expressing the isoxaben and thiazolidinone-resistant CS enzyme may be introduced into a wide variety of plants, both monocotyledon and dicotyledon, including maize, wheat, soybean, tobacco, cotton, tomatoes, potatoes, Brassica species, rice, peanuts, petunia, sunflower, sugar beet, turfgrass, etc. The gene may be present in cells or plant parts including callus, tissue, roots, tubers, propagules, plantlets, seeds leaves, seedlings, pollen, or the like.

By providing for isoxaben and thiazolidinone-resistant plants, a wide variety of formulations may be employed for protecting crops from weeds, so as to enhance crop growth and reduce competition for nutrients. The mutant CS gene can be introduced into plants and regenerated to produce a new family of transgenic plants, which possess increased resistance to isoxaben and thiazolidinone as compared with that possessed by the corresponding wild plants. Isoxaben or a thiazolidinone could be used by itself for postemergence control of weeds with transgenically protected crops, such as sunflower, soybeans, corn, cotton, etc., or alternatively, in combination formulations with other products.

Formulations could include other additives, such as detergents, adjuvants, spreading agents, sticking agents, stabilizing agents, or the like. The formulations may either be wet or dry preparations, including flowable powders, emulsifiable concentrates and liquid concentrates, such as are known in the art. The herbicidal solutions may be applied in accordance with conventional methods, for example, by spraying, irrigation, dusting, or the like.

The following is further exemplary of the invention, and specifically defines preferred techniques for the production of an isoxaben and thiazolidinone herbicide resistant *Arabidopsis thaliana*, cellulose synthase gene, a process for conferring isoxaben and thiazolidinone herbicide resistance to plants other than *Arabidopsis thaliana*, and isoxaben and thiazolidinone herbicide resistant transgenic plants.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES AND MATERIALS AND METHODS

1. Plant and seed material.

Seeds from homozygous isoxaben-resistant mutants *ixr*1-1(DH47) and *ixr*1-2 (DH48) (Heim et al., 1989) were obtained from the Arabidopsis Biological Resource Center, Columbus, Ohio (ABRC stock numbers CS 6201 and CS 6202). Seeds were vernalized for five days in 0.15 % water agar at 6°C prior to planting in a commercial peat-vermiculite-perlite mix (ProMix HP with a 1cm top-layer of ProMix PGX, Premier Horticulture Inc., Red Hill, PA, Canada). Plants were grown in the greenhouses of Carnegie Institution of Washington, Department of Plant Biology, Stanford, California between July 1998 and June 1999. Seeds from self-pollinated *ixr*1-1 and *ixr*1-2 mutants were collected separately and used for further experiments.

2. Isoxaben-resistance pilot screen.

Isoxaben (95%, N-3-[1-ethyl-1-methyl-propyl]-5-isoxazolyl-2, 6-dimethoxybenzamide) was graciously supplied by DowElanco (Indianapolis, IN) and was made up as a concentrated dimethylsulfoxide (DMSO) stock solution and used at concentrations ranging from 0.03 to 10.0 µM. The final concentration of DMSO was always 0.025%. Control cultures without isoxaben contained the same amount of DMSO as the isoxaben-treated cultures; no effect of DMSO was noted.

In order to determine a useful isoxaben concentration, which makes it possible to clearly distinguish *ixr*1-1-, *ixr*1-2- and wild-type *Arabidopsis* plants in our growth-conditions, seeds of the different genotypes were treated and plants grown as above and sprayed every 3 days, starting at the seedling stage, with 1 ml / 6 cm^2 soil surface 0.1, 0.3, 1.0 or 3.0 µM isoxaben dissolved in water containing 0.025 % (v/v) dimethylsulfoxide. This experiment showed that a concentration of 1.0-3.0 µM isoxaben in the application solution is high enough to readily allow discrimination of resistant and susceptible plants.

3. Genetic crosses.

Young flowers of isoxaben-resistant *ixr*1-1 and *ixr*1-2 mutant plants were carefully emasculated (removal of all six immature stamens) to prevent self-pollination of these flowers. After two days non-aborted, stamen-less flowers were cross-pollinated with

mature pollen from *Arabidopsis thaliana* Landsberg erecta (Ler) wild-type. F1-seeds from each mature dry silique were carefully collected, vernalized and planted as described above and the F1-plants grown and allowed to self-pollinate. F2-seeds from each *ixr*1 x Ler F1-plant were collected separately.

4. Growth analyses of F1- and F2-plants.

Seeds from wild-type Col and Ler, *ixr*1-1 and *ixr*1-2 homozygous mutants and F1- and F2-seeds were germinated again and treated as described using 2.0 µM isoxaben in the solution. Wild-type plants didn't grow at all, homozygous mutants grew well (*ixr*1-1 grew better than *ixr*1-2). All heterozygous mutant F1-plants had clearly reduced growth as compared to the homozygous mutants and F2-plants from the *ixr*1-2 x wild-type Ler showed a clear 1:2:1 segregation, as expected, in their growth behavior.

5. Growth of the mapping population.

Due to the clear growth segregation pattern of F2-plants from the *ixr*1-2 x wild-type Ler cross after treatment with 2.0 µM isoxaben, approximately 102 mg of these F2-seeds (ca. 5000 seeds) were planted in 80 150 cm^2 pots (60-65 seeds per pot). An average of 14-16 well growing plants were obtained after 2.0 µM isoxaben-treatment, fitting precisely with the expected number of F2-plants homozygous for the *ixr*1 locus. 1056 (11x96) of these plants were labeled and all other plants removed from the pots. After the plants started to bolt, tissue samples (one small cauline leaf or one small inflorescence per plant) were collected in 96-well PCR-plates (MJ Research, Cambridge, MA) and stored at -20°C until use for DNA-preparation. From all recombinant plants (see below) a second tissue sample (young leaves and flower buds) was collected one week later as a back-up and to prepare higher quality CTAB-DNA.

6. Genomic DNA-preparation.

Alkaline Lysis protocol (Klimyuk et al., 1993): 50 µl 0.25 N NaOH were added to each of the 1056 plant tissue samples collected in 96-well PCR plates (see above) on ice, and up to nine samples simultaneously ground with the upper end of polyethylene stirring rods (Sarstedt, Nümbrecht, Germany). After heating the samples in a thermocycler (model

PTC-100, MJ Research) for 30 sec at 96°C, 50 µl of 0.25 N HCl and 25 µl 0.5 M Tris-HCl (pH 8.0), 0.25% Nonidet P-40 were added and again heated to 96°C for two minutes. Afterwards the samples were directly used for PCR with SSLP-markers or stored at –20°C until use.

CTAB-protocol: 400 µl 2 x CTAB-buffer (2% (w/v) cetyl-trimethyl-ammonium bromide, 100 mM Tris-HCl pH 8.0, 20 mM EDTA, 1.4 M NaCl, 1% (w/v) polyvinyl-pyrrolidone (MW 40,000) were added to approx. 50 mg, in liquid nitrogen crushed, plant-material, mixed and incubated for 30-60 min at 65°C. 400 µl chloroform were added, the mixture vigorously mixed during 15 sec and then spun for 10 min, at 13,000 x g. The clear supernatant was carefully removed, transferred to a new sterile microtube and the DNA precipitated by addition of one volume 95% ice-cold isopropanol. After one hour at –20 °C the DNA was pelleted by centrifugation at 13,000 x g, 10 min. The supernatant was removed and the pellet washed with 70% ice-cold ethanol and allowed to dry at room temperature. The DNA-pellet was dissolved in 100 µl 10mM Tris-HCl pH 8.0, 1 mM EDTA and the DNA-concentration determined.

7. Genetic mapping of the *ixr1* locus.

Heim et al.,(1989) mapped the *ixr1* locus with phenotypic markers to the top arm of chromosome V of the *Arabidopsis* genome, approximately 3 cMorgan from the marker *lutescens*. Using southern blotting with restriction fragment length polymorphism (RFLP) markers the locus was subsequently mapped between chromosome V RFLP-markers g3715 and m217, approximately 3.7 cMorgan proximal to the former and 1.5 cMorgan distal to the latter ©. Somerville, unpublished). Using the chromosome V sequence information from the public Kasuza *Arabidopsis* data base and the RFLP-mapping data, the following two simple sequence length polymorphism (SSLP) markers were generated close to g3715 and m217.

SSLP-marker med24.2 (approx. 300 kb proximal of g3715 on the physical map of chromosome V, is directed against a $(TG)_{13}$ dinucleotide repeat on chromosome V BAC-clone MED24. Two oligonucleotide primers (F: 5' CGAACTTGAGACCTCTTGA 3' (SEQ ID

NO:7); R: 5' GCTTACCTGGAGACAGTCA 3' (SEQ ID NO: 8)) were designed with the Oligo Primer Analysis Software, version 5.0 (National Biosciences, Plymouth, MN) to PCR-amplify a 124 bp fragment from genomic wild-type Columbia DNA, containing the $(TG)_{13}$ dinucleotide repeat. Using the same primers a shorter PCR-product (<120 bp) is obtained from wild-type Landsberg genomic DNA, whereas a Col x Ler heterozygous plant gives both products, as can be easily seen on 4% agarose-gels. PCR-conditions for SSLP-marker med24.2 : 50 mM KCl, 10 mM Tris-HCl (pH 9.0 @ 25°C), 0.1% Triton X-100, 200 µM dATP, dGTP, dTTP, dCTP (each), 5 pmoles primer F, 5 pmoles primer R, 2.0 mM $MgCl_2$, 1.0 Units *Taq*. Polymerase (Promega, Madison, WI), 10-50 ng genomic DNA, final volume 20 µl. PCR-program: 1 min 94°C; 40 cycles (20 sec 94°C, 20 sec 55°C, 40 sec 72°C), 3 min 72°C.

SSLP-marker moj9.2 (approx. 100 kb proximal of m217 on the physical map of chromosome V;

molecular target:	$(TA)_{19}$ dinucleotide repeat (bases 53618-53655) on chromosome V BAC-clone MOJ9
PCR primers:	F: 5' CATGATCCATCGTCTTAGT 3'(SEQ ID 9); R: 5' AATATCGCTTGTTTTTGC 3' (SEQ ID NO: 10)
PCR-product size:	179 bp in Col, ca.160 bp in Ler
PCR-conditions:	as for med24.2 with 2.2 mM $MgCl_2$.

In all 1056 plants (=2112 chromosomes) 52 recombinants (heterozygotes for the marker) were found for med24.2 placing it 2.46 cMorgan north of *ixr*1on the genetic map of chromosome V. For the SSLP-marker moj9.2 39 recombinants were found in 2112 chromosome placing this marker 1.85 cMorgan south of *ixr*1. The DNA of one plant was recombinant for both markers. New tissue samples from all 90 recombinants were collected and genomic DNA prepared using the CTAB-protocol (see above). These higher quality DNA-preparations were subsequently used for mapping of the *ixr*1-locus with six closer markers (see Figure 1), two additional SSLP-markers muk11.1 and k18i23.1, that were identified for the purpose, and the published SSLP-markers nga158 and nga225

(Bell et al, 1994), as well as the cleaved amplified polymorphic sequence (CAPS) marker PAI2 (Bender and Fink 1995) and a CAPS-marker named MUG13E, that was identified for the purpose.

SSLP-marker muk11.1 (approx. 500 kb proximal of med24.2 on the physical map of chromosome V.

molecular target:	$(GA)_{38}$ dinucleotide repeat (bases 57187-57252) on chromosome V BAC-clone MUK11
PCR primers:	F: 5' TCCAAAGCTAAATCGCTAT 3' (SEQ ID NO: 11)
	R: 5' CTCCGTCTATTCAAGATGC 3' (SEQ ID NO: 12)
PCR-product size:	177 bp in Col, ca.120 bp in Ler
PCR-conditions:	as for med24.2

SSLP-marker nga158 (approx. 500 kb distal of moj9.2 on the physical map of chromosome V.

molecular target:	$(CT)_{14}$ dinucleotide repeat (bases 19384-19411) on BAC-clone MJJ3
PCR primers:	F: 5' ACCTGAACCATCCTCCGTC 3' (SEQ ID NO: 13)
	R: 5' TCATTTTGGCCGACTTAGC 3' (SEQ ID NO: 14)
PCR-product size:	108 bp in Col, 104 bp in Ler
PCR-conditions:	as for med24.2, except that the annealing temperature in the PCR was raised to 60°C

After this first round of convergence 13 recombinants remained on the distal side and 11 recombinants remained on the proximal side of the *ixr*1-locus. The earlier mentioned double-recombinant was not heterozygous for nga158.

SSLP-marker nga225 (approx. 36 kb proximal of muk11.1 on the sequencing map of chromosome V.

molecular target: imperfect $(GA)_{21}$ dinucleotide repeat (bases 12203-12244) on chromosome V BAC-clone MUG13

PCR primers: F: 5' TCTCCCCACTAGTTTTGTGTCC 3' (SEQ ID NO: 15)
R: 5' GAAATCCAAATCCCAGAGAGG 3' (SEQ ID NO: 16)

PCR-product size: 119 bp in Col, 189 bp in Ler.

PCR-conditions: as for med24.2, except that $MgCl_2$ was 1.75 mM.

CAPS-marker MUG13E (approx. 60 kb proximal of nga225, on the right end of BAC-clone MUG13 on the sequencing map of chromosome V.

molecular target: *Acc*I restriction enzyme site, which is present in the sequence of ecotype Landsberg erecta, but not Columbia.

PCR primers: F: 5' GATTTCCCCAGACGATTT 3' (SEQ ID NO: 17)
R: 5' AGTTTATTTGTTGCGGTTTT 3' (SEQ ID NO: 18)

PCR-product size: 2033 bp fragment (bases 79,441-81,473 of BAC MUG13) in Col and Ler before *Acc*I-digest. 1228 and 805 bp fragments in Ler after digest.

PCR-conditions: as for PAI2-marker, but annealing temperature was 54°C.

*Acc*I-digest: 7 µl of PCR-product were mixed with 9 µl H2O, 2 µl 10x buffer M (Amersham), 2 µl BSA (1mg/ml) and 1U *Acc*I, and digested for 5 hrs at 37°C. 10 µl of each digest were analyzed on a 1.2% agarose gel.

CAPS-marker PAI2 (approx. 33 kb distal of nga158 on the physical map of chromosome V.

molecular target: *Afl*III restriction enzyme site, which is present in PAI2-gene from ecotype Columbia, but not Landsberg erecta.

PCR primers: F: 5' CAGTTAATGAAACAAGCTTTGTTC 3' (SEQ ID NO: 19)
R: 5' GTTGAGAAAATCACTTTGGTG 3' (SEQ ID NO: 20)

PCR-product size: 645 bp fragment (bases 45928-46572 on BAC clone MOP10) in Col and Ler before *Afl*III-digest. 590 and 55 bp fragments in Col after digest.

PCR-conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9.0 @ 25°C), 0.1% Triton? X-100, 250 µM dATP, dGTP, dTTP, dCTP (each), 5 pmoles primer F, 5 pmoles primer R, 2.5 mM $MgCl_2$, 1.0 Units *Taq*. Polymerase (Promega), 40-50 ng genomic DNA, final volume 25 µl. PCR-program: 1 min 94°C; 35 cycles (20 sec 94°C, 20 sec 58°C, 90 sec 72°C), 3 min 72°C.

*Afl*III-digest: 7 µl of PCR-product were mixed with 9 µl H2O, 2 µl 10x buffer H (Amersham), 2 µl BSA (1mg/ml) and 1U *Afl*III, and digested for 5 hrs at 37°C. 10 µl of each digest were analyzed on a 2.5% agarose gel.

SSLP-marker k18i23.1 (approx. 101 kb proximal of PAI2 on the sequencing map of chr, V.

molecular target: purine-rich stretch (bases 17830-17870) on chromosome V BAC-clone K18I23. Length polymorphism was detected by comparative sequencing of the Ler and Col genomic sequence of that region.

PCR primers: F: 5' TGGTTAGATTTGCTGTT 3' (SEQ ID NO: 21)
R: 5' ATTCTGCATTATTAGTTGTC 3' (SEQ ID NO: 22)

PCR-product size: 139 bp in Col, 133 bp in Ler

PCR-conditions: as for med24.2, except that $MgCl_2$ was 2.5 mM and the annealing temperature during PCR was lowered to 48°C.

After convergence with these four markers three recombinants remained on the distal side and another three recombinants remained on the proximal side of the *ixr*1-locus. The region between MUG13E and k18i23.1 is approx. 53 kb in size and is spanned by three BAC-clones (MUG13, K2A11, K18I23). Proximal of MUG13E there are no predicted genes left on BAC-clone MUG13. Distal of k18i23.1 on annotated BAC-clone

K18I23 there are no obvious candidate genes that explain the resistance of the *ixr*1-mutants towards the cellulose biosynthesis inhibitor isoxaben. However, A BLAST-N search of the small not annotated (as of 8-14-99) BAC K2A11 (29.3 kb, GENBANK accession # AB018111 (SEQ ID NO: 23)) revealed a gene with perfect identity of its exons (as predicted by GRAIL, GENSCAN 1.0 and NetPlantGene) to the *Ath*-B-mRNA (GENBANK accession # AF027174 (SEQ ID NO: 24), which encodes a cellulose synthase. It is therefore concluded that the genomic cellulose synthase sequence on BAC-clone K2A11 encodes the Ath-B mRNA.

Amplification and sequencing of the cellulose synthase gene from wild-type Columbia, *ixr*1-1 and *ixr*1-2 mutant genomic DNA

Genomic DNA for each genotype was prepared from a mixture of young growing leaves and inflorescence tissue using the CTAB-protocol (see above). Six oligonucleotide primers were then designed (Oligo Primer Analysis Software, version 5.0) to amplify three (A,B,C) overlapping PCR-fragments, spanning the entire coding sequence of this cellulose synthase gene on BAC-clone K2A11 (see Figure 2).

Fragment A was amplified with primers
F_a (5'-TTAGCCATCCCAAGATTCT-3')(SEQ ID NO: 25) and
R_a (5'-CTTCAAGGGGTCAACAGTA-3')(SEQ ID NO: 26) giving a 2034 bp PCR-product (bases 13939-15972 on K2A11).

Fragment B
F_b (5'-TACCGAGCGTTTTTCCTAT-3')(SEQ ID NO: 27)
R_b (5'-CCAGCACCTAAGTTTCACA-3')(SEQ ID NO: 28)
2064 bp PCR-product (bases 12382-14445 on K2A11).

Fragment C
F_c (5'-GTTCAGTTCCCACAAAGATT-3')(SEQ ID NO: 29)
R_c (5'-TCATTCCGACCAAAAGTT-3')(SEQ ID NO: 30)
2395 bp PCR-product (bases 10620-13014 on K2A11).

PCR-conditions: 50 mM KCl, 10 mM Tris-HCl (pH 9.0 @ 25°C), 0.1% Triton? X-100, 250 µM dATP, dGTP, dTTP, dCTP (each), 10 pmoles primer $F_{a,b,c}$, 10 pmoles primer $R_{a,b,c}$, 2.5 mM $MgCl_2$, 2.0 Units *Taq*. Polymerase (Promega), 0.2 Units proofreading *Pfu*-Polymerase, 70-80 ng genomic DNA, final volume 50 µl. PCR-program: 1 min 94°C; 35 cycles (20 sec 94°C, 20 sec 55°C, 2 min 72°C), 3 min 72°C. Although a proofreading *Pfu*-Polymerase was added in the PCR, the fragments, which revealed the *ixr*1-1 and *ixr*1-2 point-mutations were amplified two more times and re-sequenced with the appropriate primers (see Figure 2, primers F10, R9, R10) to demonstrate that the mutations were not due to misincorporation of a dNTP during early cycles of PCR.

50 µl PCR-product were mixed with 10 µl 6x loading dye and loaded on a 1.2% agarose gel. All reactions yielded just one ethidium-bromide stained band when visualized under UV-light. These bands were cut out and the gel slices transferred in a microcolumn (Wizard miniprep, Promega) and spun 5 min at 8,000 x g and 5 min at 13,000 x g. The eluate was collected in a microtube and the residual in the gel slice remaining DNA was isolated using the QIAEX-kit (QIAGEN, Hilden, Germany). The collected DNA was then precipitated at -20°C after adding 20 µg glycogen, 300 mM sodium-acetate pH 5.0 and 45% isopropanol. After one hour the DNA was pelleted by centrifugation at 13,000 x g for 10 min. The supernatant was removed and the pellet washed briefly with 70% ice-cold ethanol and allowed to dry at room temperature. Finally the DNA-pellet was dissolved in 50 µl 10mM Tris-HCl pH 8.0, 0.1 mM EDTA and the DNA-concentration determined.

20 sequencing primers (F1-F10, R1-R10, 18-21-mers) were designed (Oligo Primer Analysis Software, version 5.0) equally spread over both strands in the coding region of the candidate cellulose synthase gene (see Figure 2). Sequencing reactions were set up by mixing 8 µl Big-Dye™ Dideoxy-terminator reaction-mix (PE-Applied Biosystems, Foster City, CA), 3.2 pmoles sequencing primer and 70-90 ng of the appropriate gene-cleaned PCR-product in a final volume of 20 µl. PCR-program: 1 min 94°C; 25 cycles (20 sec 94°C, 20 sec 50/55/58°C (depending on the sequencing primer), 4 min 60°C). 20 µl 2 mM $MgCl_2$ and 55 µl 95% ethanol were then added to each 20 µl sequencing reaction and the ssDNA allowed to precipitate for 30-60 min at room

temperature. After centrifugation (13,000 x g, 15 min) the supernatant was quantitatively removed, the tubes with the lids open allowed to dry and the ssDNA dissolved in 20 µl template suppression reagent (PE-Applied Biosystems), incubated 10 min at 65°C, carefully vortexed, denatured 2 min at 94°C and then stored on ice until injection in an ABI 377 automated sequencer (PE-Applied Biosystems). Sequence files were analyzed using the ABI-Prism Sequence Navigator and Sequence Analysis software (PE-Applied Biosystems).

Results

The homozygous *ixr*1-1 and *ixr*1-2 mutant plants are 300 and 90 times, respectively, more resistant to isoxaben than wild type plants (Heim et al., 1989). *ixr*1-1 (*ixr*1-2 was not tested) is also more resistant to a new thiazolidinone herbicide (TZ, compound 1). At 12 µM the thiazolidinone herbicide kills wild type *Arabidopsis*, but reduces growth of the *ixr*1-1 mutant by only 50% (Sharples et al., 1998).

The *ixr*1-2 mutation was mapped with high resolution to a small region on the top arm of chromosome V as described in materials and methods. The mapping results indicated that the *ixr*1 mutations mapped very near to a gene, *Ath*-B, encoding cellulose synthase. A cDNA clone for the *Ath*-B mRNA has previously been described by Arioli et al. (1998). The nucleotide-sequence of this clone and the deduced amino acid sequence were deposited in GenBank as accession number AF027174(SEQ ID NO: 24). Comparison of the sequence of the cDNA to the genomic sequence present on BAC clone K2A11 (GenBank accession number AB018111(SEQ ID NO: 23) indicates that the cDNA clone has 87 nucleotides at the 5' end that are not present in the genomic sequence. This extra sequence corresponds to a 59 nucleotide multiple cloning site (G *GACTC GCGCGC CTGCAG GTCGAC ACTAGT GGATCC* AAA *GAATTC* G *CGGCCG* C *GTCGAC* (SEQ ID NO: 31), restriction enzyme sites are shown in italics) that was introduced during cloning of the cDNA and an additional 28 nucleotide fragment of DNA (TACGGCTGCGAGAAGACGACAGAAGGGG)(SEQ ID NO: 32) that was also introduced at some stage during the cloning of the cDNA (see bottom middle insert in Figure 2). A search of GenBank indicated that this sequence is also found at the 5' ends of other cDNA clones; thus it is a common artifact in some libraries.

The first nucleotide of the mRNA that corresponds to the genomic clone is nucleotide 15966 of BAC K2A11 (GenBank accession number AB018111)(SEQ ID NO: 23). The open reading frame of the gene begins at nucleotide 15688 (start codon) of BAC K2A11 and ends at nucleotide 10999 (stop codon). The genomic clone contains an intron in the 5' non-translated leader sequence that corresponds to nucleotides 15736 to 15845 of BAC K2A11 and another 13 introns which divide the coding sequence in 14 exons (Figure 2), as predicted by GRAIL, GENSCAN 1.0 and NetPlantGene splice-site / exon-intron analysis. The positions of the introns and exons in the coding sequence are listed in Table 1.

In view of the evidence indicating that the mechanism of action of the isoxaben and thiazolidinone herbicides is to inhibit cellulose synthesis, this observation suggested that *Ath*-B might encode the *ixr*1 gene. To test this hypothesis, the *Ath*-B gene was cloned and sequenced from Columbia wild type and from the *ixr*1-1 and *ixr*1-2 mutants. This revealed that the *Ath*-B gene from the *ixr*1-1 mutant was identical to the wild type except for a **G** to **A** change at nucleotide 11204 on the coding (minus) strand of BAC K2A11. This mutation leads to replacement of a conserved glycine at position 998 in the *Ath*-B protein (GenBank accession number AF027174)(SEQ ID NO: 24) with an aspartic acid residue. Comparison of the nucleotide sequence of the *Ath*-B gene from wild type and the *ixr1-2* mutant indicated that they were identical except for a **C** to **T** change at nucleotide 11372 on the coding (minus) strand of BAC K2A11. This mutation resulted in replacement of a conserved threonine residue at position 942 in the Ath-B protein with an isoleucine residue. Thus, both of the *ixr1* mutations occurred in exon 14 (Fig. 2). We conclude that the *Ath*-B gene and the *IXR1* gene are identical and henceforth refer to the gene as *IXR1*.

This result teaches that either of two amino acid changes in the *IXR*1 gene renders the corresponding enzyme resistant to the inhibitory activity of the isoxaben and thiazolidinone herbicides. This invention has utility in a number of different ways.

Many plants are killed or injured by exposure to isoxaben and thiazolidinone herbicides. In order to render these plants resistant to these herbicides it is possible to introduce the herbicide resistant forms of the *IXR1* gene described herein into susceptible plants in such a way that the gene is stably inherited and expressed at a sufficient level so that it confers resistance to the herbicides. Plants can be obtained which are isoxaben and

thiazolidinone-resistant and can be grown in the field in the presence of isoxaben and thiazolidinone without significant adverse effect on their growth.

In addition to directly using the modified genes described herein, it is possible to create similarly modified forms of the gene from other species so that these genes have equivalent utility. Thus, the *IXR1* gene could be cloned from another plant by any of the methods commonly used to isolate orthologous genes from distantly related plant species. The mutations corresponding to those described herein could then be introduced into these orthologs and the modified orthologs then used to confer isoxaben and thiazolidinone resistance. This mutant gene can also be used as a selection marker in plant transformation systems with its native promoter in dicots and with a modified promoter in monocots. The exact method used to introduce the gene into a particular species of plant will vary from one species to another.

An important use of the subject invention is to facilitate the design of novel herbicides that act on cellulose synthase. One kind of application, in this respect, is to use computer programs to model the secondary, tertiary and quaternary structure of the *IXR1* wild type and mutant proteins based on the amino acid sequences of the gene products. By using such computer programs it is possible to model how the known isoxaben and thiazolidinone herbicides interact with the protein and also to model why the mutations revealed herein provide resistance to these and related compounds. This knowledge may be used to design variants of known herbicides that are active at lower rates of application or have other useful properties such as high rate of uptake by the plant, low non-plant toxicity, rapid turnover in soil or any number of other qualities that are associated with the most useful herbicides. By comparing the known or predicted structures of the *IXR1* gene from many different plant species, it may be possible to develop an understanding of how to design novel chemical inhibitors that would have broad or narrow specificity for certain classes of plants.

A related approach is to directly obtain the higher order structure of the IXR1 protein. Because the IXR1 protein is thought to be membrane associated, it may be technically challenging to obtain high resolution structural information by methods such as X-ray crystallography. However, because of parallel advances in analytical methods such as NMR, it may eventually be possible to obtain a higher order structure for use in such

purposes. The *IXR1* gene could be expressed in various alternative hosts systems in conjunction with site-directed mutagenesis to study the mechanisms by which different groups of herbicides inhibit CS. Alternatively, the system could be used to characterize the sites involved in regulation, catalysis, and herbicide binding. Binding studies may be possible on polypeptide subfragments of the protein and may not depend on the protein having overall catalytic activity.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity and understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims without departing from the spirit and scope of the invention.

Having described the basic concept of the invention, it will be apparent to those skilled in the art that the foregoing detailed disclosure is intended to be presented by way of example only, and is not limiting. Various alterations, improvements, and modifications are intended to be suggested and are within the scope and spirit of the present invention. Additionally, the recited order of the elements or sequences, or the use of numbers, letters or other designations therefor, is not intended to limit the claimed processes to any order except as may be specified in the claims. Accordingly, the invention is limited only by the following claims and equivalents thereto.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.

Table 1: Positions on BAC K2A11 and sizes of introns and coding exons of *IXR*1 in the coding region, as predicted by GRAIL, GENSCAN 1.0 and NetPlantGene. The positions of the first and last nucleotide of the start and stop codon, respectively, are shown in bold.

	Exon		Intron	
#	position on K2A11	size (nt)	position on K2A11	size (nt)
1	**15688**-15665	24	15664-15493	172
2	15492-15297	196	15296-15179	118
3	15178-14973	206	14972-14880	93
4	14879-14777	103	14776-14665	112
5	14664-14487	178	14486-14379	118
6	14378-14112	267	14111-14012	100
7	14011-13666	346	13665-13586	80
8	13585-13448	138	13447-13360	88
9	13359-13234	126	13233-13153	81
10	13152-12940	213	12939-12814	126
11	12813-12552	262	12551-12389	163
12	12388-12186	203	12185-12090	96
13	12089-11739	351	11738-11584	155
14	11583-**10999**	585		

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